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(54) Title: A METHOD FOR DETECTING AND QUANTIFYING ANALYTES BY MEANS OF SCANNING FORCE MICROSCOPY

(57) Abstract

Analytes are detected and quantified without the use of labels. A solution containing a substance capable of forming an immunocomplex is brought into contact with a support surface on which the other component of the immunocomplex to be formed is immobilized and the formed complex is detected and distinguished from substances which do not form part of the immunocomplex by means of scanning force microscopy.

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A method for detecting and quantifying analytes by means of scanning force microscopy

The present invention relates to a method for detecting and optionally quantifying analytes which are capable of forming immunocomplexes. More particularly the invention relates to such a method wherein no labelling of substances is used and wherein the immunocomplexes are formed on a support surface and then detected and optionally quantified by means of scanning force microscopy.

The most sensitive analytical techniques available today for detecting immunoassay complexes are radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). Both of these techniques are used for example for testing for drug abuse. The RIA-test is very sensitive but has the disadvantage that a radioactive labelling must be used. In ELISA testing procedures detection is made by a simple colorimetric assay. The ELISA procedure is, however, usually less sensitive than the RIA procedure.

Scanning force microscopy (SFM) for three-dimensional imaging of macromolecules was introduced in 1986 and has since then found increasing interest and use in the investigation of biomolecules. The two modes of SFM, the contact mode (CM) and the more gentle tapping mode (TM), have for example been used for investigation of the coiling of DNA, the structure of human serum albumin etc, adsorbed on support surfaces.

According to the present invention it has been found that it is possible to detect and optionally quantify analyte molecules which are capable of forming immunocomplexes by forming the complexes on a support surface and then detecting and quantifying the formed immunocomplexes by means of SFM.

As mentioned above the RIA-procedure requires labelling with radioactive substances and in other biotechnical processes labelling of substances with e.g. gold or fluorescent compounds for detection is often used. The present invention is especially advantageous in that it provides an ultrasensitive technique for detecting immunocomplexes in which no labelling of substances is required. Extremely small amounts of substances can be detected, and even single analyte mole-

cules.

The invention thus relates to a method as defined in the appended claims.

The present method is based on the finding that it is possible to detect and quantify analytes which are capable of forming immunocomplexes using SFM but without use of any special labelling for the detection.

The term "analyte" as used herein is intended to encompass the antigen or the hapten which is to be detected. The term "immunocomplex" as used herein is intended to encompass complexes formed by an antigen and an antibody or by a hapten and an antibody. Haptens are low molecular weight substances which can react with the effector cells of the immune response (humoral antibodies or stimulated T-lymphocytes). As some examples of haptens can be mentioned drugs and drug metabolites, hormones etc.

In the present method the immunocomplexes are formed by bringing a solution containing a substance capable of forming an immunocomplex into contact with a support surface on which the other component of the immunocomplex to be formed is immobilized. Hereby either analytes or antibodies against an analyte can be immobilized on the surface. In the first case, when analytes are immobilized, the solution which is brought into contact with the support surface thus contains antibodies. Usually, and preferably, antibodies will be immobilized on the support surface and a solution containing the analyte brought into contact with this. The solution can for example be a body fluid which is to be analyzed for the presence of medicaments or drug abuse. Other instances wherein the present method can be used are for example in analysis of waste fluids, for example from industrial processes, which are to be investigated with regard to toxic substances such as phenol, styrene, acrylic acid, thiazoles etc.

One way of quantifying the analytes is to let the surface with the immobilized antibody directed to the analyte gradually pass through the sample. Depending on the amount of analyte in the liquid sample immunocomplexes will be formed over varying areas of the support surface or along different distances on this, which can be used for quantitative diag-

nosis of the analyte.

The surface on which the detection is made is preferably organised in such a manner that it can accommodate a maximum number of immunocomplexes and this can for example be carried out by means of a multipoint applicator on a nanometer scale or by masking the surface to allow maximum number of complexes to be adsorbed on or bound to the surface.

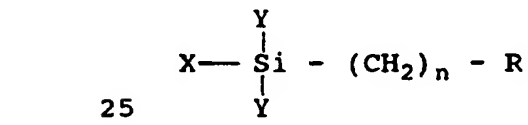
When a sample is to be analyzed the solution containing the analyte is brought into contact with the surface, on which the other component of the immunocomplex to be formed is immobilised, in such a manner as to wet the surface. It might often be necessary to use very highly diluted samples. The actual operation of the scanning force microscopy in tapping mode can be carried out in air or a liquid cell can be used. In the latter operation manner the tip of the instrument is inserted in the liquid cell which is then placed on the sample with a sealing O-ring between the cell and the sample substrate. As in the tapping mode in air the sample is mounted on top of the scanner. Since the fluid medium tends to damp the cantilevers normal resonant frequency, the entire liquid cell can be oscillated to drive the cantilever into oscillation. Further operation is similar to the operation of the tapping mode in air, which is well known to the man skilled in the art. The liquid cell is equipped with an inlet- and outlet tube which makes it possible to inject different solutions while operating the microscope.

For SFM-techniques it is important that the solid support surface is extremely smooth and plane to prevent the surface from interfering with the complexes to be analyzed. The support surface can be organic or inorganic. As examples of organic support surfaces can be mentioned teflon® and polystyrene. Usually inorganic supports are used. A support surface of glass can be used but usually smoother flat support surfaces of mica, graphite, gypsum, polished silicon, silicon wafers, or various forms of crystals which form an atomically flat surface are employed. Mica is a very suitable support surface. It has a surface roughness of ≈ 1Å (Ångström) and a new clean surface can be exposed simply be peeling off a few layers of mica for example with Scotch tape.

Generally speaking the surfaces used as supports in SFM-techniques have to be specially treated, for example by extremely careful washing, rinsing, drying etc, and often by chemical modification, in order that the desired imaging will be obtained on a level that allows separation of molecules, aggregates, complexes etc.

It is possible to utilize the present method with support surfaces as above without derivatization, i.e. chemical modification, whereby haptens, antigens or antibodies will be immobilized on the surface through adsorption. It is, however, preferred that the support surfaces are derivatized so that a covalent bonding is obtained and thus a higher reliability in the detection and quantifying. It is likewise preferred to use the tapping mode SFM for a higher reliability since there is a risk that the contact mode SFM will push away compounds and complexes, particularly of higher molecular weight, from the support surface.

The support surfaces can be derivatized by per se known reagents as used for example for inorganic support materials for chromatography. A common method is silanization. The general structure of the silane reagent for covalent immobilization of proteins on inorganic surfaces is



wherein

X and Y = Cl or X = Cl and Y = H; or

X and Y = alkyl or alkoxy groups with 1 or 2 carbon atoms;

n = 1 - 8, preferably 3 and

R is a functional group to which the naturally reactive groups of proteins can bind, such as amino or thiol groups of the protein or thiol groups introduced into the protein. Suitable functional groups R are $-CH=CH_2$, an epoxy group, NH_2, SH, pyridine or S-pyridine. Commercially available silanes can usually be chemically modified to change the functional group R to the in each case appropriate functional group, if required. Several protocols for silanization are known from the literature.

The reagent for derivatization of the surface can also be

a hydrocarbon compound corresponding to the above given formula, ie of the same formula but with a carbon atom instead of the silicon atom. Another way of modifying the support surface is by covalent binding of organic polymers to the surface. Thus examples of immobilization of proteins via polymers such as polyethylene glycol and dextran are known from the literature. These known methods must usually be optimized and modified for use with SFM.

As mentioned above several protocols for silanization are known and also for other types of derivatization. Derivatization of mica can for example be carried out in liquid or in vapours. In one liquid process a piece of mica is peeled on both sides and put in a freshly prepared solution of the derivatizing reagent in a suitable solvent, such as for example toluene. The modified mica is then rinsed with the same solvent and dried, for example using a flow of nitrogen. Methods for derivatization in vapours for different reagents have been described. A piece of mica can for example be peeled on both sides and placed in a desiccator which also contains a small amount of the reagent and the desiccator is then placed under vacuum for a certain time.

Example 1

In this experiment the formation of biocomplexes, or the lack thereof, between single human serum albumin molecules (HSA) and single or multiple antibodies, rabbit anti-human serum albumin (a-HSA), and human immunoglobulin G (IgG) adsorbed on mica surfaces was investigated. SFM probing and studies were made with TM-SFM (Nanoscope III®, Digital Instruments Inc., Santa Barbara, CA, USA) using tips with an end-radius of about 10 nm.

HSA, a-HSA and IgG were dissolved in tris buffer (pH 7.4) at concentrations of 2.5 µg/ml, 13.4 µg/ml and 0.7 µg/ml respectively. The concentrations were selected to give roughly the same area density of molecules (40-60 per μm^2) adsorbed on the surface. In each given exposure a 50 µl volume of the protein solution was placed on a freshly-cleaved mica surface (Muscovite green mica from Asheville-Schoonmaker Mica Co. Newport News VA, USA). The solution was spread out over approximately 1 cm^2, allowed to remain on the surface for 5 min-

utes and subsequently rinsed away with 1 ml of tris buffer. The surface was then dried using a flow of nitrogen and probed with TM-SFM.

To study the antigen-antibody interaction the mica surfaces were first exposed to one protein solution, rinsed with 1 ml tris buffer, dried and studied by TM-SFM. The surfaces were then exposed to the second protein solution (without recleaving), rinsed, dried and studied again by TM-SFM. Each TM-SFM image covered an area of 0.5 μm x 0.5 μm. The height histograms drawn on basis of the different TM-SFM images showed large differences which made it possible to quantitatively distinguish between different species on the surfaces. This is shown in the Table below which gives peak positions and full widths at half maximum (FWHM).

Table

Experiment	1st Peak pos. ± FWHM (nm)	2nd Peak pos. ± FWHM (nm)
A HSA	0.62±0.28	
B a-HSA	1.91±0.66	
C IgG	1.75±0.60	
D HSA exposed to IgG	≈0.62	≈1.75
E HSA exposed to a-HSA	≈0.62	3.03±1.60
F IgG exposed to HSA	≈0.62	1.35±0.72
G a-HSA exposed to HSA	≈0.62	1.38≈0.80

* Note: In experiments E, F and G the HSA contribution has been subtracted for the 2nd peak.

The following remarks are given to further clarify the results given in the Table above.

Although the height information obtained by TM-SFM is not totally topological, the height histogram showed large differences which allowed quantitative distinction between the presence of different species on the surfaces. In experiment D, wherein HSA preadsorbed on the mica surface was exposed to IgG two distinct populations of molecules, within height ranges corresponding to those of separately adsorbed HSA and IgG were observed, and no interaction between HSA and human IgG could be observed. In experiment E, wherein HSA on mica

was exposed to a-HSA, larger features, both with regard to lateral dimensions and height, were observed than with either HSA or a-HSA absorbed separately on mica. The image showed the expected complexing, since the a-HSA is raised in rabbit to specifically interact with HSA. In the results shown for experiment E the HSA contribution for the 2nd peak has been subtracted.

The experiments further showed that HSA binds spontaneously and irreversibly to hydrophilic mica surfaces which were not derivatized, even though the net charge of HSA as well as the surface charge are negative. IgG did, however, bind more weakly to the mica and would thus preferably be immobilized on a derivatized surface.

Claims

1. A method for label-free detecting and optionally quantifying of analytes, characterized in that a solution containing a substance capable of forming an immunocomplex is brought into contact with a support surface on which the other component of the immunocomplex to be formed is immobilized and that the formed complex is detected and distinguished from substances which do not form part of the immunocomplex by means of scanning force microscopy.

2. A method according to claim 1, characterized in that the two components forming the immunocomplex are antibodies and antigens.

3. A method according to claim 1 or 2, characterized in that the solution contains the substance to be detected and that this an antigen.

4. A method according to claim 1, characterized in that the solution contains the substance to be detected and that this a low molecular drug or a hormone.

5. A method according to any of the preceding claims, characterized in that the support surface is of glass, mica or polished silicon.

6. A method according to claim 5, characterized in that the support surface is chemically modified for immobilization by covalent binding.

7. A method according to claim 6, characterized in that the support surface is silanized.

8. A method according to any of the preceding claims, characterized in that tapping mode scanning force microscopy is used for the detection.

9. A method according to any of the preceding claims, characterized in that the scanning force microscope is operated in air.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/00431

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/543 // G01B 7/34
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, US PATFULL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	Proc.Natl.Acad.Sci., Volume 92, February 1995, (USA), Sándor Damjanovich et al, "Structural hierarchy in the clustering of HLA class I molecules in the plasma membrane of human lymphoblastoid cells", page 1122 - page 1126, See"materials and methods", page 1122-1123 --	1

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

01/04/96

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